ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2004, p. 143–150 Vol. 48, No. 1
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.1.143–150.2004
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Molecular Basis of Intrinsic Macrolide Resistance in the *Mycobacterium tuberculosis* Complex

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Received 7 July 2003/Returned for modification 28 July 2003/Accepted 8 October 2003

The intrinsic resistance of the *Mycobacterium tuberculosis* complex (MTC) to most antibiotics, including macrolides, is generally attributed to the low permeability of the mycobacterial cell wall. However, nontuberculous mycobacteria (NTM) are much more sensitive to macrolides than members of the MTC. A search for macrolide resistance determinants within the genome of *M. tuberculosis* revealed the presence of a sequence encoding a putative rRNA methyltransferase. The deduced protein is similar to Erm methyltransferases, which confer macrolide-lincosamide-streptogramin (MLS) resistance by methylation of 23S rRNA, and was named ErmMT. The corresponding gene, *ermMT* (*erm37*), is present in all members of the MTC but is absent in NTM species. Part of *ermMT* is deleted in some vaccine strains of *Mycobacterium bovis* BCG, such as the Pasteur strain, which lack the RD2 region. The Pasteur strain was susceptible to MLS antibiotics, whereas MTC species harboring the RD2 region were resistant to them. The expression of *ermMT* in the macrolide-sensitive *Mycobacterium smegmatis* and BCG Pasteur conferred MLS resistance. The resistance patterns and ribosomal affinity for erythromycin of *Mycobacterium* host strains expressing *ermMT*, *srmA* (monomethyltransferase from *Streptomyces ambofaciens*), and *ermE* (dimethyltransferase from *Saccharopolyspora erythraea*) were compared, and the ones conferred by ErmMT were similar to those conferred by SrmA, corresponding to the MLS type I phenotype. These results suggest that *ermMT* plays a major role in the intrinsic macrolide resistance of members of the MTC and could be the first example of a gene conferring resistance by target modification in mycobacteria.

The *Mycobacterium* genus comprises more than 70 species, including the major human pathogens responsible for tuberculosis (*Mycobacterium tuberculosis*, *Mycobacterium africanum*, and *Mycobacterium bovis*) and leprosy (*Mycobacterium leprae*). This genus also includes soil saprophytes and water microorganisms, some of which (e.g., those belonging to the *Mycobacterium avium* complex) can cause opportunistic infections, especially in immunocompromised patients. Mycobacteria are intrinsically resistant to most commonly used antibiotics and chemotherapeutic agents. Due to its specific structure and composition, the mycobacterial cell wall is an effective permeability barrier, considered to be a major factor in promoting this natural resistance (25). Only a few drugs are active against mycobacteria, and the emergence of multidrug-resistant *M. tuberculosis* strains is becoming a major problem worldwide (16).

Macrolides inhibit protein synthesis in a wide range of bacteria by binding to the large ribosomal subunit (18, 23). Like other protein synthesis inhibitors that affect the large subunit, they can also prevent the formation of the 50S particle in growing cells (8). Natural macrolides, such as erythromycin, are not effective against mycobacteria, but semisynthetic derivatives, such as clarithromycin and azithromycin, have stronger antimycobacterial activities and are widely used to treat infections caused by some nontuberculous mycobacteria (NTM) (13, 46, 55). However, these semisynthetic macrolides are not effective for the treatment of tuberculosis because of the natural resistance of *M. tuberculosis* (41, 50). The reason for this difference in macrolide resistance between *M. tuberculosis* and NTM is not understood. It may be related to differences in cell wall permeability, but the synergistic contribution of a specific resistance mechanism might be required.

Macrolide resistance mechanisms have been characterized for a broad range of gram-positive and gram-negative bacteria, including pathogenic isolates and macrolide producers. The most widespread mechanism involves the methylation of an adenine residue within the 23S rRNA, conferring resistance to macrolide, lincosamide, and streptogramin (MLS) antibiotics (26, 42, 53). Other macrolide resistance mechanisms involve antibiotic inactivation, active drug efflux, and mutated ribosome components (ribosomal proteins or 23S rRNA) (27, 54). In NTM, mutations in the 23S rRNA gene are the only acquired macrolide resistance mechanism to have been characterized so far (51). Since mycobacteria possess only one or two rRNA operons, mutations in one of them is sufficient to confer a resistance phenotype (44). Macrolide resistance is frequently acquired during macrolide therapy due to 23S rRNA mutations (34, 36, 52).

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
M. tuberculosis H37Rv		ATCC 27294
M. bovis		ATCC 19210
M. africanum		ATCC 25420
M. microti		ATCC 19422
M. bovis BCG Moreau	Vaccine strain (Brazil), RD2 region not deleted	ATCC 35736
M. bovis BCG Pasteur	Vaccine strain, RD2 region deleted, host for DNA cloning	no. 1173 P_2; Pasteur Institute
M. smegmatis mc^2 155	Highly transformable mutant, host for DNA cloning	47
E. coli DH5α	Host for DNA cloning	Clontech
Plasmids		
pMIP12	Kan^r; high-copy number *E. coli-Mycobacterium* shuttle vector	28
pIJ43	Amp^r; source of *ermE*	48
pOS41.33	Amp^r, source of *srmA*	39
pOMV2	Amp^r; PCR (EMT1, EMT2) fragment containing *ermMT*, cloned into pUC18	This study
pOMV16	Kan^r; *Bam*HI-*Kpn*I PCR (EMT7, EMT8) fragment containing *ermMT*, cloned into pMIP12	This study
pOMV20	Kan^r; *Bam*HI-*Kpn*I PCR (ERME1, ERME2) fragment containing *ermE*, cloned into pMIP12	This study
pOMV30	Kan^r; *Bam*HI-*Kpn*I PCR (SRMA1, SRMA2) fragment containing *srmA*, cloned into pMIP12	This study

[a] Amp^r, ampicillin resistance; Kan^r, kanamycin resistance.

The complete genome sequences of two *M. tuberculosis* strains, H37Rv (6, 10) and CDC1551 (17), the one from *M. bovis* (19), and partial sequence data for *M. tuberculosis* 210 are now available. The *M. leprae* genome is also completely sequenced (11). Other mycobacteria are currently being sequenced. Among the mycobacterium species for which genome sequence data are available, some are macrolide resistant whereas others are macrolide sensitive. We used these sequence data to search for candidate genes that could explain the difference in susceptibility to macrolides. We report that one of these candidates, encoding a putative 23S rRNA methyltransferase, confers macrolide resistance in sensitive hosts and greatly contributes to the intrinsic macrolide resistance of the *M. tuberculosis* complex (MTC).

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The strains and plasmids used in this study are listed in Table 1.

Escherichia coli DH5α, the strain used for cloning experiments, was grown in liquid or on solid Luria-Bertani medium (Gibco) at 37°C. *Mycobacterium smegmatis* was grown at 37°C with shaking in Middlebrook 7H9 broth (Difco) containing 0.2% (vol/vol) glycerol or on solid Middlebrook 7H10 medium (Difco) containing 0.5% (vol/vol) glycerol. MTC strains were grown in the same conditions as *M. smegmatis*, except that the liquid broth was supplemented with 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco) and 0.05% (vol/vol) Tween 80 (7H9-OADC-Tween). When required, antibiotics were added at the following concentrations: ampicillin, 50 $\mu g \cdot ml^{-1}$; kanamycin, 50 $\mu g \cdot ml^{-1}$ for *E. coli* and 15 $\mu g \cdot ml^{-1}$ for mycobacteria. Mycobacterial strains were maintained on Löwenstein-Jensen medium (Bio-Rad).

In silico analysis. Genome sequences were searched using the BLAST program (1). The query sequences included the following: (i) 23S rRNA methyltransferases conferring macrolide resistance by target modification e.g., ErmE (GenBank accession no. CAB60001) from *Saccharopolyspora erythraea*, a canonical Erm protein acting at position A2058 in 23S rRNA, and TlrB (accession no. CAB37345) from *Streptomyces fradiae*, acting at position G748 in 23S rRNA; (ii) macrolide-inactivating enzymes, such as glycosyltransferases, e.g., MgtA (accession no. Q54387) from *Streptomyces lividans* and OleI (accession no. AAC12648) from *Streptomyces antibioticus*, phosphotransferases, e.g., MphA (accession no. BAB12239) from *E. coli* or MphBM (accession no. BAA34540) from *Staphylococcus aureus*, and esterases, EreA (accession no. AAA25640) and EreB (accession no. CAA27626) from *E. coli*; (iii) efflux pumps, e.g., various ABC transporters, SMR (small multidrug resistance) proteins, or major facilitators. Preliminary sequence data were obtained from The Institute for Genomic Research website at http://www.tigr.org for *M. avium* and *M. smegmatis*, from the Sanger Institute web site for *Mycobacterium marinum* (http://www.sanger.ac.uk/Projects/M_marinum/blast_server.shtml), and from the Center for Computational Genomics and Bioinformatics at the University of Minnesota web site (http://www.ccgb.umn.edu/) for *Mycobacterium paratuberculosis*.

Antimicrobial agents and determination of MICs. The MICs of all antibiotics were determined by using the BACTEC radiometric method (Becton Dickinson) for MTC strains at pH 7.3 as previously reported (40). The MICs of all drugs were determined in three independent experiments and were highly reproducible. For *M. smegmatis*, MICs were determined by the standard agar macrodilution method on Mueller-Hinton medium supplemented with 5% OADC (15).

In vitro DNA manipulations. All restriction endonuclease digestions, ligations, DNA modifications, and PCR amplifications were performed according to standard protocols (43). Oligonucleotides EMT1 and EMT2 were used to amplify a 1.3-kb DNA fragment containing the *ermMT* open reading frame and flanking regions from *M. tuberculosis* H37Rv (EMT1, 5′CACTGAGGCTCGCCGACT, EMT2 5′GCAGAGAAGGATGCCGCT). This 1.3-kb fragment was cloned into pUC18 to yield pOMV2. The oligonucleotides EMT7 and EMT8 (EMT7, 5′TCAGGATCCGCCCTCGGACGGTCGCG, *Bam*HI site underlined; EMT8, 5′GCAGGTACCTCCGGGGTTTCGGTTATTTGGTAGC, *Asp*718I site underlined) were used to amplify the *ermMT* coding region cloned into pOMV2 and to introduce convenient restriction sites for cloning in the expression vector pMIP12. The resulting plasmid was named pOMV16. The oligonucleotides ERME1 and ERME2 were used to amplify the *ermE* coding region and to introduce convenient restriction sites for cloning in the expression vector pMIP12 (ERME1, 5′AGCGGGATCCAGTTCGGACGAGCAGCCG, *Bam*HI site underlined; ERME2, 5′GCCGACATCAACCTCTGGTACCTCTCGACC, *Asp*718I site underlined). *srmA* was amplified using oligonucleotides SRMA1 and SRMA2 (SRMA1, 5′TTAGGATCCCGCCCCACCCAGCGTG, *Bam*HI site underlined; SRMA2, 5′TGAGGTACCGTGCGCGGCGATGGAG, *Asp*718I site underlined). The resulting fragment was cloned into pMIP12 in the same manner as *ermMT* and *ermE*. The oligonucleotides RD21 and RD22 were used to check for the RD2 deletion in *M. bovis* BCG strains (RD21, 5′AGCACGAGATCGGCCGCGACAAGC; RD22, 5′ GGAAGAGCTGCCATGGGTGAGGCGA). These two primers are complementary to sequences located immediately upstream and downstream of RD2 and give a PCR product of 347 bp when RD2 is absent. The oligonucleotides EMT13 and EMT14 were used to amplify *ermMT* homologues from *M. africanum* and *Mycobacterium microti* (EMT13, 5′GCGAAGGATCCTGCCAGGTGGGTCTAGTGGGT; EMT14, 5′

TTGGGTCTAGAGGTACCTCCGGGGTTTCGGTTATTTGG). The sequences of amplified fragments were determined using the ABI Prism BigDye terminator kit (PE Biosystems) and an automatic sequencer (ABI Prism 310) as recommended by the manufacturer.

Bacterial transformation. *E. coli* was transformed according to standard protocols (43). Electrocompetent *M. smegmatis* mc^2155 (47) cells were prepared as described previously (24, 47). For electroporation, approximately 1 μg of DNA was mixed with 100 μl of competent cells in 0.2-cm chilled electroporation cuvettes and put on ice for 5 min. Electroporation was performed using a Gene Pulser apparatus (Eurogentec) set at 2.5 kV, 40 μF, 200 Ω with a single pulse. The cells were immediately diluted with 1 ml of Middlebrook 7H9 medium containing 0.2% (vol/vol) glycerol. After 2 h at 37°C with vigorous shaking, cells were plated on Middlebrook 7H10 plates containing the appropriate antibiotics for selection of the transformants. After 3 days at 37°C, single colonies were picked.

Electrocompetent *M. bovis* BCG cells were prepared as described previously (37). One microgram of DNA was mixed with 200 μl of competent cells and subjected to electroporation using the same conditions as for *M. smegmatis*. Then, 5 ml of 7H9-OADC-Tween 80 was added, and the culture was incubated for 48 h at 37°C with shaking to allow the expression of antibiotic resistance genes. Cells were then plated on 7H10 medium supplemented with 10% OADC and appropriate antibiotics. Transformants were picked after 3 weeks at 37°C.

Preparation of plasmid and genomic DNA. Standard methods were used to extract plasmid DNA from *E. coli* (43) and mycobacteria (24). The cetyltrimethylammonium bromide method was used to extract total DNA from mycobacteria (2).

Preparation of ribosomes and erythromycin binding essay. *M. smegmatis* or *M. bovis* BCG (200 ml) was grown at 37°C to late log phase, harvested by centrifugation (6,000 × *g*, 10 min, 4°C), and washed twice with buffer A (10 mM Tris-HCl [pH 7.2], 4 mM $MgCl_2$, 10 mM NH_4Cl, 100 mM KCl). Pellets were frozen and then resuspended in buffer A to a final volume of 10 ml. Cells were broken by two passages through a French pressure cell at 124 MPa, with the addition of 2 μg of DNase I (RNase free)/ml^{-1} between the two passages. Ribosomes were isolated by successive centrifugations: lysates were first centrifuged at 30,000 × *g* for 30 min at 4°C to remove cell debris and unbroken cells, and the supernatant was then centrifuged at 100,000 × *g* for 2 h at 4°C to pellet the ribosomes. Finally, ribosomes were resuspended in buffer A overnight on ice and stored at −80°C in small aliquots. Erythromycin binding was determined as previously described (15).

RESULTS

Search for determinants of macrolide resistance in mycobacteria. *M. tuberculosis* genes that confer moderate levels of macrolide resistance when overexpressed have already been described. These genes, encoding proteins belonging to the SMR (*mmr* (14) or ABC family (*drrA* and *drrB* (9) of transporters, are highly conserved in all strains from the MTC, but their homologues are also present in macrolide-sensitive strains, such as *M. leprae*, *M. avium*, and other NTM. This suggests that these genes do not play a crucial role in high-level macrolide resistance.

The available mycobacterial sequence data were BLAST searched for sequences similar to those of various proteins involved in macrolide resistance. Whenever possible, these sequences were chosen from *Actinomycetales* (including macrolide-producing organisms), a group of bacteria comprising the *Mycobacterium* genus. Several gene products with moderate similarity (below 40% identity and 50% similarity) to some macrolide resistance proteins were detected, but they were generally present in both macrolide-sensitive and -resistant species. The most interesting hit, found with ErmE from *S. erythraea* as a query sequence, was a gene named *Rv1988* in *M. tuberculosis* H37Rv. The deduced protein presents similarity to 23S rRNA methyltransferases of the Erm family. The sequence of this gene was identical in the three *M. tuberculosis* strains (H37Rv, CDC1551 [gene *MT2042*], and 210) and also in *M. bovis* AF2122/97 (gene *Mb2010*). Using the primers EMT13 and EMT14 deduced from the *M. tuberculosis* sequence, we PCR amplified homologous genes from *M. africanum* and *M. microti*, both belonging to the MTC. The sequences of these amplified products were identical to that of *M. tuberculosis*/*M. bovis* (data not shown). BLAST searches failed to detect homologous genes in *M. leprae* and *M. avium* or in the partially sequenced *M. smegmatis*, *M. paratuberculosis*, and *M. marinum* genomes. Our attempts to detect homologues of *Rv1988* in some NTM, including uncharacterized clinical resistant isolates, by either hybridization or PCR, were unsuccessful (data not shown).

BCG is a live attenuated bacterial vaccine derived from *M. bovis*. *M. bovis* and the *M. bovis* BCG strains differ by several deletions (3, 22, 31). One of these regions of difference, RD2, is absent in certain BCG strains, such as BCG Pasteur, and present in others, such as BCG Moreau. This deletion precisely affects *Mb2010* (Fig. 2). We used PCR to verify that the complete *Mb2010* coding sequence was present in the BCG Moreau strain. PCR, using the primers RD21 and RD22, confirmed that the BCG Pasteur strain used in this study lacked the RD2 region (and part of *Mb2010*).

***ermMT*, a putative 23S rRNA methyltransferase gene in the *M. tuberculosis* complex.** The 179-amino-acid long protein deduced from *Rv1988* presents similarity with the Erm protein family, members of which confer macrolide, lincosamide, streptogramin B resistance by methylation of the 23S rRNA at a highly conserved adenine nucleotide (A2058 [*E. coli* numbering]). This protein is 36% identical and 50% similar to LmrB from the lincomycin producer *Streptomyces lincolnensis* (accession no. AAB23456) and 34% identical and 47% similar to ErmE from the erythromycin producer *S. erythraea*. The *Rv1988* gene and the identical coding sequences in the MTC were therefore called *ermMT*. This gene was also assigned the name *erm(37)* according to the new nomenclature system proposed for classification of *erm* genes (42) (see also http://faculty.washington.edu/marilynr/). ErmMT possesses the characteristic signature of rRNA adenine methyltransferases (Pfam accession no. PF00398). ErmMT was aligned with some other proteins from the Erm family (Fig. 1). Eight conserved motifs (numbered from I to VIII) (Fig. 1) are common to *S*-adenosylmethionine (SAM)-dependent methyltransferases, which methylate the exocyclic amino groups of adenine and cytosine (32). Structural studies of the Erm proteins ErmAM and ErmC′ (5, 45, 56) have shown that motifs I to III and V are part of the SAM-binding site. The glycine-rich motif I (amino acids 40 to 44 of ErmMT) binds the methionine in SAM and constitutes the fingerprint of SAM-dependent methyltransferases. Residues from motifs IV and VI to VIII form a pocket adjacent to the SAM-binding site that probably binds the target adenine. All of these motifs are present in ErmMT.

MLS resistance profile of members of the MTC. If *ermMT* plays a role in macrolide resistance, we would expect a correlation between the MLS resistance profiles of the various strains of the MTC and the presence of this gene. To test this hypothesis, we determined the in vitro activity of MLS antibiotics against members of the MTC, including two BCG strains, the Pasteur strain with the RD2 region deleted ($ermMT^-$) and the Moreau strain with this region intact ($ermMT^+$). MICs were determined for several macrolides, including 14-mem-

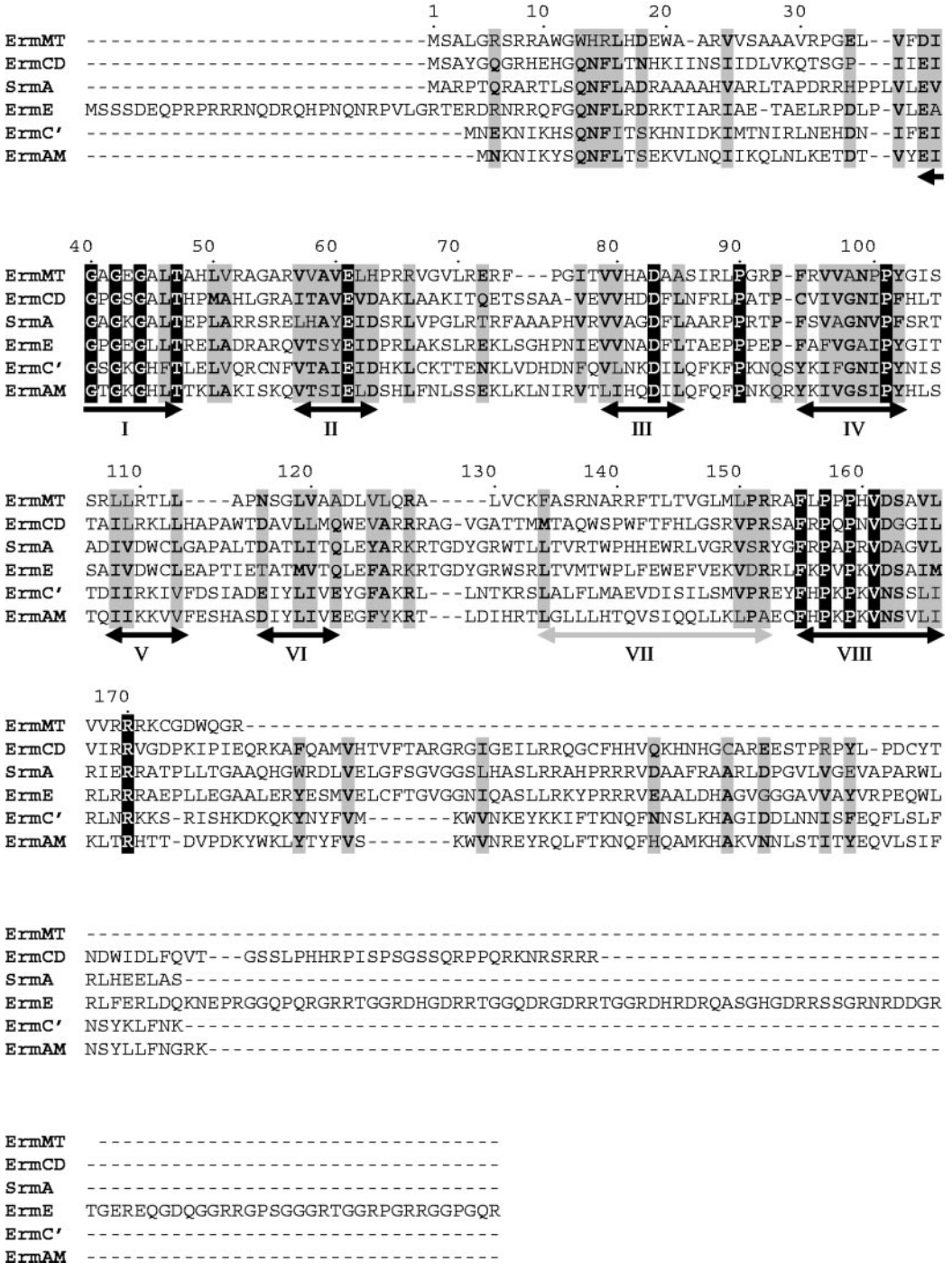

FIG. 1. Comparison of the amino acid sequence of ErmMT (Erm37 subgroup, accession no. CAA98396) with those of ErmCD (ErmX subgroup) from *Corynebacterium diphtheriae* (accession no. AAA98484), SrmA (ErmO subgroup) from *Streptomyces ambofaciens* (accession no. CAA11706), ErmE (ErmE subgroup) from *Saccharopolyspora erythraea* (accession no. CAB60001), ErmC′ (ErmC subgroup) from *Bacillus subtilis* (accession no. AAA98136), and ErmAM (ErmB subgroup) from *Streptococcus pneumoniae* (accession no. S12727). The structures of ErmC′ and ErmAM are known (Protein Data Bank accession no. 2ERC and 1YUB). The alignment was made using the MultAlign program (12). Conserved regions are shaded (grey, moderate conservation; black, identical residues). The positions and approximate extents of the eight conserved motifs found in other DNA and RNA methyltransferases are indicated by arrows. Motif VII is weakly conserved in the Erm family.

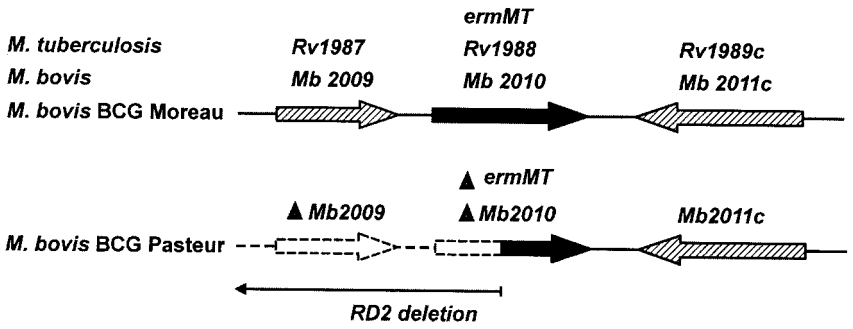

FIG. 2. Schematic representation of the *ermMT* regions of *M. tuberculosis*, *M. bovis*, and *M. bovis* BCG Moreau compared to that of *M. bovis* BCG Pasteur (RD2 deletion).

bered ring structures (erythromycin, its derivatives clarithromycin and roxithromycin), 15-membered ring structures (azithromycin), 16-membered ring structures (spiramycin), and for lincosamide (clindamycin) and streptogramin (pristinamycin) (Table 2). The two streptogramin components were tested together. The new streptogramin quinupristin-dalfopristin was also tested and gave the same results as pristinamycin (data not shown). Two resistance profiles were clearly distinguishable within the MTC: *M. tuberculosis* H37Rv, *M. bovis*, *M. africanum*, *M. microti*, and the BCG Moreau strain showed a low level of resistance to clarithromycin and pristinamycin (MIC of >2 μg/ml and ≤ 32 μg/ml) but a high level of resistance to other macrolides and clindamycin (MIC of ≥ 32 μg/ml). *M. bovis* BCG Pasteur was much more sensitive to all antibiotics tested (MIC of ≤ 32 μg/ml). With the exception of pristinamycin, the MICs for the BCG Pasteur strain were 8 to 256 times lower than those for the other members of the MTC. Our results are in agreement with a study showing that ermMT$^-$ *M. bovis* BCG strains (strains Pasteur, Denmark, and Glaxo) are susceptible to clarithromycin, whereas *M. tuberculosis*, *M. bovis*, and *M. africanum* are resistant (41).

The other MTC strains were much more resistant to all MLS antibiotics than *M. bovis* BCG Pasteur. This is consistent with target modification rather than with differences in specific transport system or drug inactivation mechanisms. MLS ribosomal resistance could be due to rRNA changes (i.e., mutations or modifications). Mutations of the rRNA conferring MLS resistance are mostly clustered in the central loop of domain V in 23S rRNA (51). The sequence of 23S rRNA in this region (from nucleotides 1923 to 2771 [*E. coli* numbering]) was determined for *M. bovis* BCG Pasteur, *M. africanum*, and *M. microti* (data not shown). In all cases, the sequences were identical to the published sequences for *M. tuberculosis* and *M. bovis*, eliminating the possibility that the domain V rRNA mutation plays a role in the resistance phenotype. Therefore, the correlation between the presence of *ermMT* and the MLS resistance phenotype strongly suggests that *ermMT* plays a role in the intrinsic MLS resistance of mycobacteria belonging to the MTC. Due to the similarity of ErmMT to methyltransferases that modify 23S rRNA, we decided to investigate the existence of ribosomal modification.

TABLE 2. MICs of MLS antibiotics against reference strains from the MTC

Strain	MIC (μg/ml)[a]						
	ERY	CLR	ROX	AZM	SPI	CLI	PRI
M. tuberculosis	256	16	128	256	512	≥2,058	32
M. bovis	128	2	32	128	1,024	≥2,058	8
M. africanum	512	16	128	256	512	≥2,058	32
M. microti	64	2	32	64	1,024	≥2,058	4
BCG Moreau	128	2	ND	ND	512	1,024	4
BCG Pasteur	4	0.25	0.5	1	32	16	2

[a] ERY, erythromycin; CLR, clarithromycin; ROX, roxithromycin; AZM, azithromycin; SPI, spiramycin; CLI, clindamycin; PRI, pristinamycin; ND, not determined.

The methylation of A2058 of 23S rRNA by Erm proteins prevents the binding of MLS antibiotics to the prokaryotic ribosomes. We studied the binding of radiolabeled erythromycin to ribosomes isolated from sensitive or resistant strains to substantiate our hypothesis on ribosomal modification. Ribosomes were isolated from *M. tuberculosis* and *M. bovis* BCG Pasteur, since these two strains differ by their MLS resistance patterns and by the presence or absence of *ermMT*. Increasing amount of ribosomes were mixed with a constant amount of radiolabeled erythromycin. For the same amount of ribosomes, at least five times less drug bound to *M. tuberculosis* ribosomes than to *M. bovis* BCG Pasteur ribosomes (data not shown; see below), suggesting ribosomal modification.

Effect of *ermMT* expression on macrolide resistance. To obtain direct evidence of the ability of *ermMT* to confer macrolide resistance, a fragment corresponding to the coding part of *ermMT* was amplified with the primers EMT7 and EMT8. After intermediate cloning steps and sequence verification, this fragment was cloned into pMIP12, an *E. coli*/*Mycobacterium* shuttle vector, to yield pOMV16. In this construction, the *ermMT* gene was located downstream of the constitutive *pBlaF** promoter from *Mycobacterium fortuitum* (28) and of properly located translation signals and upstream of a terminator.

It has been observed that dimethylation of A2058 confers the MLS type II phenotype, characterized by a high level of resistance to all MLS antibiotics, while monomethylation of A2058 confers the MLS type I phenotype, characterized by a high level of resistance to lincosamides and intermediate resistance to macrolides and streptogramins (38). In order to compare the resistance profile conferred by *ermMT* with those conferred by characterized Erm methyltransferases, the *ermE* and *srmA* genes were cloned into pMIP12, yielding, respec-

TABLE 3. MICs of MLS antibiotics against *M. bovis* BCG Pasteur harboring the empty vector (pMIP12), pOMV16 (pMIP12+*ermMT*), or pOMV20 (pMIP12+*ermE*), and *M. smegmatis* harboring the same constructions or pOMV30 (pMIP12+*srmA*)

Strain	MIC (μg/ml)[a]					
	ERY	CLR	AZM	SPI	CLI	PRI
BCG Pasteur(pMIP12)	8	0.5	ND	64	16	4
BCG Pasteur(pOMV16)	256	16	ND	512	>1,024	4
BCG Pasteur(pOMV20)	>1,024	512	ND	>1,024	>1,024	32
M. smegmatis(pMIP12)	32	0.5	4	8	512	64
M. smegmatis(pOMV16)	512	4	128	256	>1,024	128
M. smegmatis(pOMV20)	>1,024	>1,024	>1,024	>1,024	>1,024	512
M. smegmatis(pOMV30)	256	4	128	512	>1,024	64

[a] ERY, erythromycin; CLR, clarithromycin; AZM, azithromycin; SPI, spiramycin; CLI, clindamycin; PRI, pristinamycin; ND, not determined.

tively, pOMV20 and pOMV30. The *ermE* gene encodes a dimethyltransferase (49), while *srmA* encodes a monomethyltransferase (39), both enzymes acting on A2058 in the 23S rRNA.

These plasmids (pOMV16, pOMV20, pOMV30, and pMIP12) were introduced into the macrolide-sensitive strains, *M. bovis* BCG Pasteur and *M. smegmatis*, and the resulting strains were tested for MLS resistance (Table 3; MICs not determined for pOMV30 in *M. bovis* BCG Pasteur). The MICs show that *ermMT* expression leads to MLS resistance of the host. The resistance levels of *M. bovis* BCG Pasteur expressing *ermMT* were comparable to those of the other MTC strains without a deletion of *ermMT*. Thus, the deletion of *ermMT* from *M. bovis* BCG Pasteur may be sufficient to explain the MLS sensitivity of this strain. The MICs for strains expressing *ermMT* were lower than those associated with *ermE* expression and comparable to those associated with *srmA* expression, indicating that ErmMT confers a type I MLS resistance.

Effect of *ermMT* expression on ribosomes. Ribosomes were isolated from *M. smegmatis* strains harboring pOMV16 (*ermMT*), pOMV20 (*ermE*), pOMV30 (*srmA*), or pMIP12 (empty vector) and tested for the binding of radiolabeled erythromycin (Fig. 3). The control strain harboring pMIP12 is sensitive to erythromycin, and accordingly the drug bound efficiently to its ribosomes. Ribosomes from the highly macrolide-resistant strain expressing *ermE* were refractory to erythromycin binding. Ribosomes from the strain expressing ErmMT bound amounts of erythromycin comparable to those bound by those modified by SrmA, but they bound more drug than those modified by ErmE. The results obtained with ribosomes extracted from *M. bovis* BCG Pasteur harboring the different plasmids were essentially the same. This is consistent with the hypothesis that ErmMT confers resistance by ribosomal modification and could, as with SrmA, monomethylate the ribosomes at position 2058 in the large rRNA.

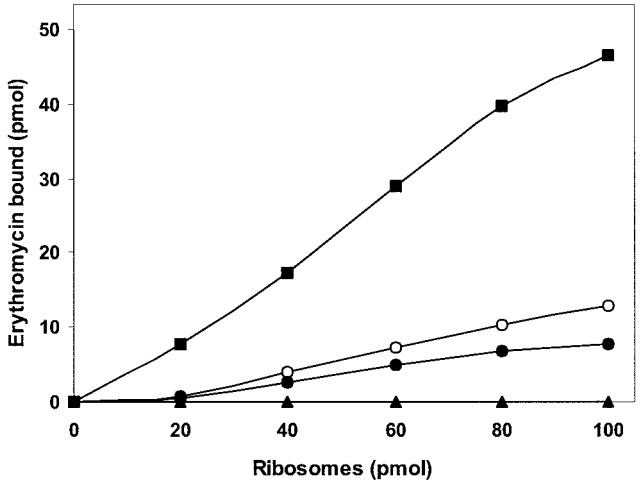

FIG. 3. Binding of erythromycin to ribosomes extracted from *M. smegmatis* strains harboring the empty vector (pMIP12, ■), pOMV16 (*ermMT*, ○), pOMV20 (*ermE*, ▲), and pOMV30 (*srmA*, ●).

DISCUSSION

Although several controversial reports have been published concerning macrolide activity against *M. tuberculosis* (7, 30, 50), we have confirmed that all species from the MTC, except some BCG strains, are intrinsically resistant to MLS antibiotics. The nature of this resistance is generally attributed to the specific structure of the mycobacterial cell wall.

Our in silico analysis suggested that *Rv1988* could be an MLS resistance gene, belonging to the *erm* family of genes encoding 23S rRNA methyltransferases. Our experimental results indicate that this gene, now called *ermMT* (*erm37*), plays an essential role in the MLS resistance phenotype of species from the MTC. The presence of *ermMT* in all MTC species indicates that it was not recently acquired and was present in the common ancestor of the MTC (4, 35). The gene *ermMT* is highly conserved in all species of the MTC, but in some BCG strains part of *ermMT* is lacking. In comparison with their parental *M. bovis* strain, the BCG vaccine strains lack one region (RD1) and are polymorphic for other deletions (RD2, RD8, RD14, RD16) (3, 22). BCG strains containing or not containing *ermMT* are used as vaccines. We have shown that the absence of the RD2 region (absence of *ermMT*) is related to susceptibility to macrolide antibiotics. This might explain the controversial results concerning the efficiency of erythromycin for the treatment of some complications of BCG vaccination (21). Even though the complications of vaccination or intravesical BCG immunotherapy are not very frequent, it might be preferable to use a strain without *ermMT*, so that it remains possible to use macrolides for treatment purposes.

Erm proteins modify the N6 amino group of adenine 2058 (*E. coli* numbering) in 23S rRNA by mono- or dimethylation, thus conferring different MLS resistance profiles. Erm dim-

ethylation is one of the major mechanisms of macrolide, lincosamide, streptogramin B resistance in pathogenic bacteria, whereas monomethylation occurs in *Actinobacteria*, which sometimes accommodate both kinds of methyltransferase. Our experiments indicate that ErmMT could be a monomethyltransferase, as inferred indirectly from the MLS type I resistance phenotype and by comparison of the level of erythromycin binding to mono- and dimethylated ribosomes.

More than 60 Erm proteins, which can be classified into 30 different classes, have been identified to date (42). ErmMT, which is 179 amino acids long, is the smallest representative of this family, the other members of which are between 243 amino acids (ErmA [accession no. A25101] from *S. aureus*) and 381 amino acids (ErmE from *S. erythraea*) long. The three-dimensional structures of two Erm methyltransferases, ErmC′ (5, 45) and ErmAM (56), have been solved. Both enzymes have a bilobal structure, consisting of a large catalytic N-terminal domain of about 180 amino acids, which binds SAM, and a C-terminal domain thought to be involved in recognition of the substrate 23S rRNA. The best-conserved regions and the eight motifs common to DNA, RNA, and small-molecule methyltransferases are located in the N-terminal domain. ErmMT aligns well with the N-terminal domain of other Erm methyltransferases (Fig. 1), but ErmMT is much shorter, so the equivalent of the C-terminal domain is totally absent. Indeed, the end of ErmMT corresponds almost exactly to the end of the N-terminal domain of ErmC′ and ErmAM. Determination of the ErmMT structure and studies on the recognition of the 23S rRNA substrate by ErmMT might lead us to reconsider the basis of 23S rRNA recognition by Erm proteins. This is in agreement with the results of a recent study suggesting that the key RNA-binding residues are located in the large catalytic domain and not in the small C-terminal domain of ErmC′ (33).

In *M. tuberculosis*, the primary mechanism of drug resistance is the mutation of target genes (20). However, we have shown that the *ermMT* gene confers high-level MLS resistance as a result of target modification, making the *M. tuberculosis* ribosomes refractory to macrolides. However, the monomethylation of A2058 only mildly affects the activity of new macrolide derivatives, the ketolides (29), even if the activities of the already developed molecules do not seem sufficient for their clinical use against the MTC (41). Another possible strategy for overcoming resistance due to ribosome methylation might be to search for inhibitors of the Erm family, which could be administered with the MLS antibiotic. In this type of approach, inhibiting the binding of Erm to the 23S rRNA might be more selective than the inhibition of the methylation reaction. Due to its small size, ErmMT might represent the minimal Erm protein and constitute a good model for this type of study.

ACKNOWLEDGMENTS

We thank Brigitte Gicquel and her collaborators, especially Nathalie Winter and Jean-Marc Reyrat, from the Pasteur Institute, for providing several cloning vectors and bacterial strains and for valuable discussions. We also thank Véronique Vincent (Pasteur Institute) for kindly providing several *Mycobacterium* strains and Luiz R. R. Castello-Branco (Fundação Ataulpho de Paiva, Rio de Janeiro, Brazil) for the kind gift of the strain *M. bovis* BCG Moreau. We also thank the whole laboratory of Microbiology (Hôpital de Versailles) for their excellent technical work.

K. Buriánková received a fellowship from the French Government and support from the Erasmus exchange program. A. Gondran received a fellowship from the "Fondation pour la Recherche Médicale." This research was supported in part by the Grant Agency of the Czech Republic, grants no. 204/00/1252 and 301/02/1469.

ADDENDUM IN PROOF

Since the submission of this article, the presence of an *erm* gene, *erm* (*38*), has been reported in *M. smegmatis* (K. A. Nash, Antimicrob. Agents Chemother. **47:**3053–3060, 2003).

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